

Atty. Dkt. No. SALK2370-2  
(088802-5455)

**Amendments to the Specification**

Please amend the specification as follows.

Please edit page 23, line 25 as follows:

**TABLE 3 (SEQ ID NO: )**

Please edit page 10, line 4 (top line of Table 1) as follows:

Action Site- Carbon Number	X Position	Y Position	Z Position	Amino Acid <u>residue</u> (SEQ ID NO:1)
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Please edit the paragraph beginning at page 178, line 20 as follows:

The CHS homodimer contains two functionally independent active sites. Consistent with this information, bound CoA thioesters and product analogs occupy both active sites of the homodimer in the CHS complex structures. These structures identify the location of the active site at the cleft between the upper and lower domains of each monomer. Each active site consists almost entirely of residues from a single monomer with Met, 3, from the adjoining monomer being the only exception. There are remarkably few chemically reactive residues in the active site. Four residues conserved in all the known CHS-related enzymes (Cys<sub>164</sub>, Phe<sub>215</sub>, His<sub>303</sub>, and Asn<sub>336</sub> **of SEQ ID NO:1**) define the active site. Cys<sub>164</sub> apparently serves as the nucleophile and as the attachment site for polyketide intermediates as previously suggested for both CHS and STS (Lanz, et al, J. Biol. Chem. 266: 9971-9976,1991). His<sub>303</sub> most likely acts as a general base during the generation of a nucleophilic thiolate anion from Cys,, since the N of His<sub>303</sub> is within hydrogen bonding distance

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of the sulfur of Cys<sub>164</sub>. Phe<sub>215</sub> and Asn<sub>336</sub> may function in the decarboxylation reaction, as discussed below. Topologically, three interconnected cavities intersect with these four residues and form the active site architecture of CHS. These cavities include a CoA-binding tunnel, a coumaroyl-binding pocket, and a cyclization pocket.

Please edit the three paragraphs beginning at page 179, line 7 as follows:

The CoA-binding tunnel is 16 angstroms long and links the surrounding solvent with the buried active site. Binding of the CoA moiety in this tunnel positions substrates at the active site, as observed in the C<sub>164</sub>S mutant of SEQ ID NO:1 (described in greater detail below) complexed with malonyl- or hexanoyl-CoA. The conformation of the CoA molecules bound to CHS resembles that observed in other CoA binding enzymes. The adenosine nucleoside is in the 2'-endo conformation with an anti-glycosidic bond torsion angle. At the tunnel entrance, Lys<sub>55</sub>, Arg<sub>58</sub>, and Lys<sub>62</sub> of SEQ ID NO:1 hydrogen bond with two phosphates of CoA. Apart from these interactions, and an additional hydrogen bond between the backbone amide nitrogen of Ala<sub>308</sub> of SEQ ID NO:1 and the first carbonyl of the pantetheine moiety, van der Waals contacts dominate the remaining interactions between CHS and CoA. The pantetheine arm of the CoA extends into the enzyme positioning the terminally bound thioester-linked substrates near Cys<sub>164</sub> of SEQ ID NO:1.

Both naringenin and resveratrol bind at the active site end of the CoA-binding tunnel. The interactions observed in the naringenin and resveratrol complexes define the coumaroyl-binding and cyclization pockets (see Figure 5). The space to the lower left of the CoA-binding tunnel's end serves as the coumaroyl-binding pocket. Residues of this pocket (Ser<sub>133</sub>, Glu<sub>192</sub>, Thr<sub>194</sub>, Thr<sub>197</sub>, and Ser<sub>338</sub> of SEQ ID NO:1) surround the coumaroyl-derived portion of the bound naringenin and

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resveratrol molecules and interact primarily through van der Waals contacts. However, the carbonyl oxygen of Gly<sub>216</sub> of SEQ ID NO:1 hydrogen bonds to the phenolic oxygen of both naringenin and resveratrol and the hydroxyl of Thr<sub>197</sub> of SEQ ID NO:1 interacts with the carbonyl of naringenin derived from coumaroyl-CoA. The identity of the residues in this pocket likely contributes to the preference for coumaroyl-CoA as a substrate for parsley CHS over other cinnamoyl CoA starter molecules, like caffeoyl-or feruloyl-CoA.

In both the naringenin and resveratrol complexes, the malonyl-derived portion of each molecule occupies a large pocket adjacent to Cys<sub>164</sub> of SEQ ID NO:1 suggesting this is where the polyketide reaction intermediate cyclizes into the new ring system and where aromatization of the ring occurs. The six-carbon chain of hexanoyl-CoA also binds in this pocket. Physically, the size of the pocket limits the number of acetate additions to three. Phe<sub>265</sub> of SEQ ID NO:1 separates the coumaroyl-binding site from the cyclization pocket and may function as a mobile steric gate during successive rounds of polyketide elongation. Although a polyketide possesses a number of hydrogen bond acceptors through which specific interactions could aid in proper folding for the cyclization reaction, the residues of the cyclization pocket, including Thr<sub>132</sub>, Met<sub>137</sub>, Phe<sub>215</sub>, Ile<sub>254</sub>, Gly<sub>256</sub>, Phe<sub>265</sub>, and Pro<sub>375</sub> of SEQ ID NO:1, provide few potential hydrogen bond donors. As in the coumaroyl-binding pocket, van der Waals contacts dominate the interaction between CHS and both naringenin and resveratrol. Thus, the surface topology of the cyclization pocket dictates how the malonyl-derived portion of the polyketide is folded and how the stereochemistry of the cyclization reaction leading to chalcone formation in CHS and resveratrol formation in STS is controlled.

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Please edit the paragraph beginning at page 185, line 2 as follows:

Absolute conservation of Cys<sub>164</sub>, Phe<sub>215</sub>, His<sub>303</sub>, and Asn<sub>336</sub> of SEQ ID NO:1 occurs in CHS-like sequences, including several bacterial proteins possessing very low (typically 20-30%) amino acid sequence identity. Moreover, all CHS-like proteins exhibit strong conservation of residues shaping the geometry of the active site (Pro<sub>138</sub>, Gly<sub>163</sub>, Gly<sub>167</sub>, Leu<sub>214</sub>, Asp<sub>217</sub>, Gly<sub>262</sub>, Pro<sub>304</sub>, Gly<sub>305</sub>, Gly<sub>306</sub>, Gly<sub>335</sub>, Gly<sub>374</sub>, Pro<sub>375</sub>, and Glu<sub>376</sub> of SEQ ID NO:1). Although the functions of the bacterial CHS-like proteins remain unknown, these enzymes likely form polyketides or polyketide-CoA thioesters in a manner resembling CHS. However, steric differences resulting from sequence variation in both the coumaroyl-binding pocket and the cyclization pocket strongly suggest alternate substrate and product specificity in the bacterial enzymes.

Please edit the paragraph beginning at page 191, line 8 as follows:

Functional diversity among other homodimeric iterative PKSs, like *p*-coumaroyltriacyclic acid synthase (CTAS), acridone synthase (ACS), and the *rppA* protein from *Streptomyces griseus*, likely results from variations of residues lining the initiation/elongation cavity. As demonstrated, positions 197, 256, and 338 of SEQ ID NO:1 distinguish between tetraketide products derived from a final Claisen condensation in wild-type CHS and triketide products derived from an enolate-directed condensation in the CHS triple mutant. Although CHS, CTAS, and ACS generate tetraketides, each enzyme differs in either the cyclization reaction or in the identity of the starter molecule. CTAS forms the same enzyme-bound tetraketide as CHS but does not catalyze the final cyclization reaction. Comparison of these two enzymes reveals that substitution of Thr 197 of SEQ ID NO:1 in CHS with an asparagine in CTAS may prevent the covalently-bound tetraketide intermediate from

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undergoing cyclization into chalcone. ACS uses N-methylanthranoyl-CoA as a starting substrate to produce the alkaloid acridone. Three differences between CHS (Thr<sub>132</sub>, Ser<sub>133</sub>, and Phe<sub>265</sub> of SEQ ID NO:1) and ACS (Ser<sub>132</sub>, Ala<sub>133</sub>, and Val<sub>265</sub>) may alter starter molecule specificity. In ACS, these changes likely widen the portion of the cavity corresponding to the *p*-coumaroyl-binding site in CHS to accommodate N-methylanthranoyl-CoA binding. Comparative changes in the active site cavity allow formation of longer polyketides. The *rppA* protein forms a pentaketide from five acetates derived from malonyl-CoA decarboxylation. Thr<sub>137</sub>, Ala<sub>138</sub>, Thr<sub>199</sub>, Leu<sub>202</sub>, Met<sub>259</sub>, Leu<sub>261</sub>, Leu<sub>268</sub>, Pro<sub>304</sub>, and Ile<sub>343</sub> of 2-PS are replaced by Cys<sub>106</sub>, Thr<sub>107</sub>, Cys<sub>168</sub>, Cys<sub>171</sub>, Ile<sub>228</sub>, Tyr<sub>230</sub>, Phe<sub>237</sub>, Ala<sub>261</sub>, and Ala<sub>295</sub>, respectively, in the *rppA* protein. Models of the *rppA* protein based on the 2-PS and CHS structures show that cavity volume is 1145 Å<sup>3</sup> in the *rppA* protein versus 274 Å<sup>3</sup> in 2-PS (or 923 Å<sup>3</sup> in CHS). Manipulation of the active site through amino acid substitutions offers a strategy for increasing the molecular diversity of polyketide formation through both the choice of starter molecule and the number of subsequent condensation steps.